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Dose-Dependency of 2-Acetylaminofluorene Binding to Liver DNA and Hemoglobin in Mice and Rats

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16. ABSTRACT The carcinogenic activity of 2-acetylaminofluorene (2-AAF) in mice and rats has been accurately described so that it is a suitable archetypical chemical for investigations of parameters of cross-species variation. The binding of orally administered (9-^{14}C)2-AAF to liver DNA in mice and rats was dose related in a linear manner between 1 to 100 umol/kg body wt. By sephadex LH-20 chromatography, it was determined that the formation of 2-AAF adducts over the dose range studied was both qualitatively and quantitatively similar. Hepatic DNA binding was greater in rats compared to mice. No sex difference was observed in the DNA binding in rats so that the higher incidence of liver cancer in male compared to female rats could not be explained by a greater amount of DNA binding in males. The binding of orally administered (9-^{14}C)2-AAF to hemoglobin in mice and rats was dose related in a linear manner between 0.1 to 100 umol/kg. The results of 2-AAF binding to DNA and hemoglobin would indicate that for an oral dose of 2-AAF, the rate of formation of reactive metabolites was similar between 0.1 and 100 umol/kg body wt. However, the sevenfold greater DNA binding and 2.5-fold greater hemoglobin binding in rats compared to mice were more than an order of magnitude less than 246-fold greater carcinogenicity of 2-AAF in rat liver. The results of the study supported a pharmacokinetic model that related the metabolism of 2-AAF to reactive metabolites to the binding of the metabolite to DNA and hemoglobin. The results do not support a relationship of macromolecular binding to carcinogenic potency.

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Dose-Dependency of 2-Acetylaminofluorene Binding to Liver DNA and Hemoglobin in Mice and Rats

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Dose Dependency of 2-Acetylaminofluorene Binding to Liver DNA and Hemoglobin in Mice and Rats. PEREIRA, M. A., LIN. L-H. C., AND CHANG, L. W. (1981). *Toxicol. Appl. Pharmacol.* **60,** 472–478. The carcinogenic activity of 2-acetylaminofluorene (2-AAF) in mice and rats has been accurately described so that it is a suitable archetypical chemical for investigations of parameters of cross-species variation. The binding of orally administered [9-^{14}C]2-AAF to liver DNA in mice and rats was dose related in a linear manner between 1 to 100 μmol/kg body wt. By Sephadex LH-20 chromatography, it was determined that the formation of 2-AAF adducts over the dose range studied was both qualitatively and quantitatively similar. Hepatic DNA binding was greater in rats compared to mice. No sex difference was observed in the DNA binding in rats so that the higher incidence of liver cancer in male compared to female rats could not be explained by a greater amount of DNA binding in males. The binding cf orally administerd [9-^{14}C]2-AAF to hemoglobin in mice and rats was dose related in a linear manner between 0.1 to 100 μmol/kg. The results of 2-AAF binding to DNA and hemoglobin would indicate that for an oral dose of 2-AAF, the rate of formation of reactive metabolites was similar between 0.1 and 100 μmol/kg body wt. However, the sevenfold greater DNA binding and 2.5-fold greater hemoglobin binding in rats compared to mice were more than an order of magnitude less than 246-fold greater carcinogenicity of 2-AAF in rat liver. The results of the study supported a pharmacokinetic model that related the metabolism of 2-AAF to reactive metabolites to the binding of the metabolites to DNA and hemoglobin. The results did not support a relationship of macromolecular binding to carcinogenic potency.

The estimation of the carcinogenicity of a chemical in humans from animal data requires the knowledge of the parameters that determine interspecies variation and the shape of the dose–response curve at the low doses expected from human exposure. The carcinogenic activity of 2-acetylaminofluorene (2-AAF) in mice and rats has been extensively described so that it is suitable for use as a model chemical in investigations of parameters of interspecies variation. The dose–response relationship of liver tumor induction by 2-AAF was determined in mice (Littlefield *et al.*, 1979) and rats (Albert *et al.*, 1972). Bioassays that employ tumor incidence as their endpoint for assessing carcinogenicity of a chemical are relatively insensitive and require large numbers of animals. Chemical carcinogens have been proposed to bind DNA either directly or indirectly after metabolic activation (Brooks and Lawley, 1964; Miller, 1979). The difference in the susceptibility of mice and rats to the carcinogenic action of 2-AAF might be explained by differences in DNA binding. The demonstration of the relationship of DNA binding to carcinogenic potency might provide a method for estimating the carcinogenicity of a chemical at low doses. In this study, we determined the dose dependency of 2-AAF binding to liver DNA for comparison to its liver carcinogenicity.

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The binding of chemical carcinogens and mutagens to hemoglobin (Hb) has been proposed as a systemic dose monitor that correlates to target organ DNA binding and carcinogenicity (Ehrenberg and Osterman-Golkar, 1980). Circulating hemoglobin can be viewed as an *in vivo* trapping agent for electrophilic metabolites of carcinogens (Farmer *et al.*, 1980; Pereira and Chang, 1980). The stability of the hemoglobin measured in months is not altered by the binding of the carcinogen (Osterman-Golkar *et al.*, 1976). Therefore, the determination of the extent of hemoglobin acylation by 2-AAF is not obscured by possible degradation of the alkylated hemoglobin. The amount of 2-AAF alkylated hemoglobin would therefore be a measure of the total systemic distribution of reactive metabolites. The distribution of reactive 2-AAF metabolites from their site of formation in the liver to the bladder has been proposed for the induction of bladder tumors (Kadlubar *et al.*, 1977). Therefore, in this study we also determined the dose dependency of 2-AAF binding to hemoglobin.

METHODS

Animals. Male and female (200 to 250 g body wt) Sprague–Dawley rats and male and female CD-1 mice (8 to 9 weeks old) were purchased from Charles River (Portage, MI) and used throughout these studies. Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) and distilled water were provided *ad libitum*.

Treatment of animals. [9-^{14}C]Acetylaminofluorene was purchased from Amersham Corp. (Arlington Heights, IL) and dissolved in corn oil. In some situations nonradiolabeled 2-AAF (Sigma Chemical Co., St. Louis, MO) was added. Immediately before use, the radiochemical purity was determined to be greater than 98% by high-performance liquid chromatography on a C-18 reverse phase ODS column (Spectra Physics, Santa Clara, CA) eluted with a linear 10 to 100% methanol in water gradient. Rats received 1 μl/g body wt and mice 2 μl/g body wt of the 2-AAF dissolved in corn oil by gavage between 8 and 9 A.M. Twenty-four hours later blood was obtained while under light ether anesthesia from rats by cardiac puncture into a heparinized syringe and from mice by retroorbital bleeding with a heparinized Pasteur pipette. The animals then were sacrificed by cervical dislocation and the liver excised.

Isolation, degradation, and column chromatography of 2-AAF bound DNA. DNA was isolated from the liver by a modification of the procedure described by Kirby (1967). The liver was homogenized in a lysing medium containing 1% sodium triisopropylnapthalene sulfonate, 6% isobutanol, 6% sodium-*para*-aminosalicylate, 1% sodium chloride, and 0.01% sodium deoxycholate and incubated with proteinase K for 1 hr. After extraction with Kirby's phenol solution, the DNA was precipitated with 2-ethoxyethanol. The DNA was then sequentially treated with RNase, α-amylase, and Pronase followed by a second phenol extraction. After 2-ethoxyethanol precipitation, the DNA was dissolved in water. Contamination by protein was less than 3% as determined by the procedure of Lowry *et al.* (1951). For determination of radioactivity, an aliquot of the DNA was hydrolyzed in 0.1 N HCl at 70°C for 30 min, dissolved in ACS (Amersham Corp., Arlington Heights, IL), and counted in a Beckman 9000 liquid scintillation counter (Beckman Instrument Co., Palo Alto, CA). DNA concentration was determined by the procedure of Kissane and Robins (1958).

The DNA was enzymatically hydrolyzed to deoxynucleosides by the sequential addition of the following enzymes: (1) nuclease P_1 (Sigma Chemical Co., St. Louis, MO) in 0.1 N sodium acetate, pH 6.0, 5–6 hr; (2) acid phosphatase from wheat germ (PL Biochemical Inc., Milwaukee, WI) with the addition of 50 mM magnesium chloride, 24 hr; and (3) alkaline phosphatase type 3R (Sigma Chemical Co., St. Louis, MO) with the addition of Tris buffer to pH 7.5, 16 hr. After concentration under nitrogen, the 2-AAF conjgated DNA was chromatographed on a Sephadex LH-20 (0.9 × 50 cm) eluted at a flow rate of 20 min/hr with (1) 0.01 M ammonium carbonate, pH 8.9 (70 ml), (2) 0.01 M ammonium carbonate in 35% ethanol (175 ml), and (3) 0.01 M ammonium carbonate in 60% ethanol (150 ml) at a flow rate of 8 ml/hr. Absorbance was monitored at 260 and 305 nm with an ISCO UV-monitor (Instrument Specialties Co., Lincoln, NE) and radioactivity determined after the addition of ACS.

Isolation, hydrolysis, and chromatography of 2-AAF conjugated hemoglobin. The 2-AAF conjugated hemoglobin was isolated and the amount of bound [9-^{14}C]2-AAF present determined as previously described (Pereira and Chang, 1980). For isolation of the 2-AAF adducts in hemoglobin, the conjugated hemoglobin, after the removal of the heme, was hydrolyzed at 110°C for 22 hr in 6 N HCl containing 0.05% 2-mercaptoethanol and 1 mg/ml of 2-mercaptosuccinic acid. The hydrolysate was evaporated to dryness in a heated vacuum desiccator (55°C) over sodium hydroxide pellets. The dry sample was dissolved in 30 mM sodium acetate (pH 5.5) in methanol (1:1), and chromatographed on a high-performance liquid chromatograph equipped with a re-

verse phase ODS column (Spectra Physics, Santa Clara, CA). The column was eluted at a flow rate of 1 ml/min with a linear gradient of 30 mM sodium acetate, pH 5.5, containing 5–100% methanol. Radioactivity was determined after the addition of an aliquot of each fraction to ACS.

RESULTS

DNA Binding

Acetylaminofluorene-conjugated DNA was obtained from the liver of rats treated with various doses of 2-AAF and sacrificed 24 hr after dosing. The DNA was enzymatically degraded to deoxyribonucleosides and chromatographed on a Sephadex LH-20 column. The elution profile of the radiolabeled products contained four major peaks (Fig. 1). Peak II cochromatographed with *N*-(deoxyguanosine-8-yl)-*N*-AAF kindly supplied by Drs. J. B. Weinstein and D. Grunberger (Columbia University, N.Y.). Peak III would appear to be eluted in the region of the elution profile where Kriek (1969) found the deacetylated moiety of 2-AAF bound to DNA. Similar to our findings, Kriek also found that this peak represented

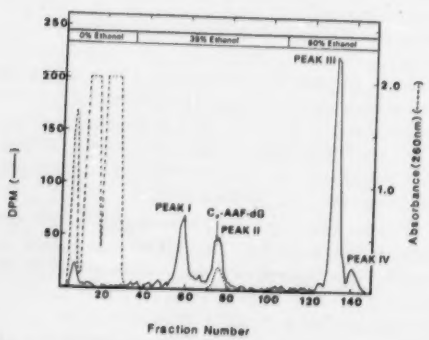

FIG. 1. Sephadex LH-20 column chromatography elution profile of [9-^{14}C]2-AAF conjugated DNA after enzyme degradation to deoxyribonucleosides. Rats were administered po 10 μmol/kg body wt and sacrificed 24 hr later. The deoxynucleosides were monitored by absorbances at 260 nm and were eluted in the eluate containing zero ethanol. The absorbance of the added *N*-(deoxyguanosine-8-yl)-*N*-AAF is also shown.

TABLE 1

RELATIVE CONTRIBUTION OF THE DIFFERENT [9-^{14}C]2-ACETYLAMINOFLUORENE ADDUCTS TO THE DNA BINDING

Dose (μmol/kg)	Peak (relation concentration)			
	I	II	III	IV
100	17[a]	18	49	8.3
10	18	14	49	5.9
1	12	11	57	6.7

[a] Percentage of the total radioactivity applied to the Sephadex LH-20 column.

over 70% of the amount of DNA alkylation. Peak I is an unidentified alkylation product that might have resulted by the breakdown of the deacetylated adduct in Peak III, since the deacetylated adduct was demonstrated by Kriek (1969) to be relatively unstable during isolation. The relative contribution of the four peaks to the total acylation of DNA was independent of dose between 1 and 100 μmol/kg body wt (Table 1). The recovery in the four peaks of the total radioactivity applied to the column was also independent of dose and greater than 90%. Therefore, the amount of the major alkylation adducts present in the four peaks was directly proportional to total DNA binding at all doses studied.

The dose dependency of the binding of 2-AAF to liver DNA was determined in animals orally administered 1, 10, or 100 μmol/kg body wt (Fig. 2). The binding to 2-AAF to liver DNA in mice and rats increased as a linear function of dose. In rats, the DNA binding was approximately sevenfold greater than mice. The dose-response relationship of DNA binding was determined in male rats and female mice, since these were the respective sexes used in the carcinogenicity bioassays. There was however no apparent sex difference in the binding of 2-AAF to liver DNA in rats; however, female mice appeared in bind 2-AAF to a greater extent than males (Table 2). The low level of bind-

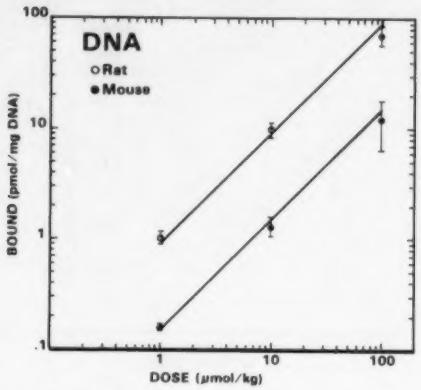

FIG. 2. Dose dependency of 2-AAF binding to liver DNA. Animals were administered po 1, 10, or 100 μmol/kg body wt [9-^{14}C]2-AAF and sacrificed 24 hr later. Results are means of five animals ± SE and the 45° line that best fits the data is shown.

ing to DNA in the liver of male mice necessitated the pooling of the livers.

Hemoglobin Binding

Acetylaminofluorene-conjugated hemoglobin obtained from rats treated with [9-^{14}C]2-AAF, after the removal of the heme, was hydrolyzed with 6 N HCl and chromatographed on a high-performance liquid chromatograph equipped with an ODS reverse phase column. The elution profile (Fig. 3) demonstrated that the radioactivity was present as metabolities of 2-AAF bound to hemoglobin. There was no evidence for incorporation of the radioactivity into amino acids by their *de novo* synthesis. Instead, greater than 95% of the radioactivity was found in the hydrophobic region of the elution profile representing metabolites of 2-AAF covalently bound to amino acids.

The dose–response relationship of 2-AAF binding to hemoglobin was determined in animals administered 0.1 to 100 μmol/kg body wt and sacrificed 24 hr later. The binding of 2-AAF to hemoglobin increased as a linear function of dose and in rats the binding was 2.5-fold greater than in mice (Fig. 4). There was no apparent sex difference in hemoglobin binding (Table 2). The efficiency of hemoglobin binding by 2-AAF was calculated from the slope of the dose–response curve, i.e., 42 and 102 pmol bound/g Hb/μmol administered/kg body wt for

TABLE 2

BINDING OF [9-^{14}C]2-ACETYLAMINOFLUORENE TO LIVER DNA AND HEMOGLOBIN[a]

Species	DNA binding index[b]	Hemoglobin binding index[c]	Carcinogenic potency (kg body wt) (day)/μmole
Mouse			
Female	0.132 ± 0.026[d]	41.7 ± 1.8	5.8×10^{-2}[e]
Male	0.036	36.7 ± 2.7	
Rat			
Female	0.947 ± 0.118	79.6 ± 7.9	
Male	0.982 ± 0.151	102.4 ± 8.4	14.3[f]

[a] Animals were administered intragastrically 10 μmol/kg body wt [9-^{14}C]2-AAF and blood obtained 24 hr later. Immediately afterward the animals were sacrificed and the liver DNA isolated.

[b] DNA binding index: pmol bound to DNA/mg DNA/μmol/kg bd wt as determined for a 10 μmol/kg dose.

[c] Hemoglobin binding index: pmol bound to Hb/g Hb/μmol/kg bd wt as determined for a 10 μmol/kg dose.

[d] Results are means of five animals ± SE except for the male mice DNA binding which is the pool of five livers.

[e] Average of the seven doses of the 24 months sacrificed with $n = 3$. Data from Littlefield *et al.* (1979).

[f] Average of the 18 mg/kg day (224 days), 18 mg/kg day (290 days), 6 mg/kg day (340 days), and 6 mg/kg day (380 days) sacrifice with $n = 3$. Data from Albert *et al.* (1972).

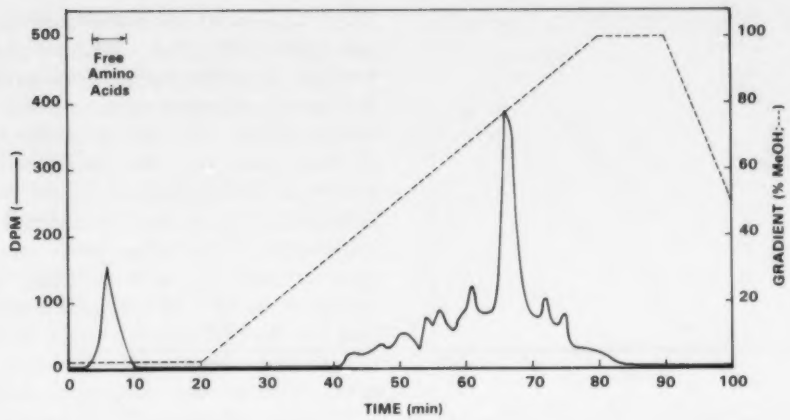

FIG. 3. High-performance liquid chromatography of [9-^{14}C]2-AAF conjugated hemoglobin after acid hydrolysis to amino acids. The rat was administered po 10 μmol/kg body wt and sacrificed 24 hr later.

mice and rats, respectively. By assuming that a rodent contains 50 ml blood/kg body wt and 14 g Hb/100 ml blood, the efficiency of 2-AAF binding to hemoglobin was calculated to be 0.03 and 0.07% of an oral dose for mice and rats, respectively.

DISCUSSION

The estimation of the carcinogenicity of a chemical in humans from animal data requires knowledge of the dose–response relationship at doses below which the determination of the tumor incidence is not practical. Pharmacokinetic models have been proposed by Gehring and Blau (1977) and Anderson *et al.* (1980) to estimate the carcinogenic response at low doses. These models relate the carcinogenic potency to the formation of a reactive metabolite (RM) that binds the receptor molecule, DNA. We have modified these models to include the binding of RM to hemoglobin (RM-Hb) so that RM can be estimated at doses below the sensitivity of the determination of RM binding to DNA (RM-DNA; Fig. 5). RM-Hb is a component of CBN (covalent binding, nongenetic) in the model described by Gehring and Blau (1977). RM can be determined by the following relationships:

$$\mathrm{RM} + \mathrm{DNA} \xrightarrow{k'_{\mathrm{DNA}}} \mathit{RM\text{-}DNA} \quad (1)$$

$$\mathit{RM} + \mathit{Hb} \xrightarrow{k'_{\mathrm{Hb}}} \mathit{RM\text{-}Hb} \quad (2)$$

$$\mathrm{RM} = \frac{[\mathrm{RM\text{-}DNA}]}{[k'_{\mathrm{DNA}}]\,[\mathit{DNA}]} = \frac{[\mathit{RM\text{-}Hb}]}{[k'_{\mathrm{Hb}}]\,[\mathit{Hb}]} \quad (3)$$

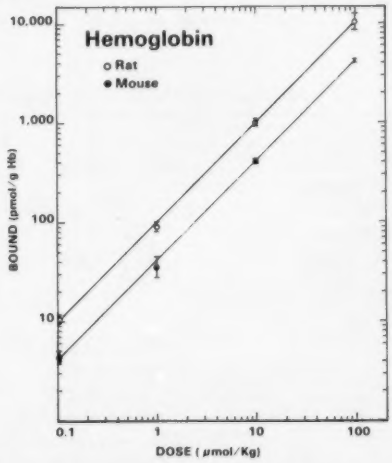

FIG. 4. Dose dependency of 2-AAF binding to hemoglobin. Animals were administered po 0.1, 1, 10, or 100 μmol/kg body wt [9-^{14}C]2-AAF and sacrificed 24 hr later. Results are means of five animals ± SE and the 45° line that best fits the data is shown.

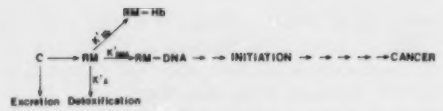

FIG. 5. A diagram of the pharmacokinetic model for the relationship between DNA binding, hemoglobin binding, and carcinogenic potency. C is dose of the carcinogen, RM is reactive metabolite, RM-DNA is the adduct of RM to DNA, and RM-Hb is the adduct of RM to hemoglobin.

The apparent rate constants, k'_{DNA} and k'_{Hb}, are modified by the effect of the migration of RM from its site of formation in the smooth endoplasmic reticulum to the nuclear DNA and the hemoglobin in the circulating erythrocytes. By this model, the binding of a carcinogen to hemoglobin can be used to estimate the binding to DNA.

The binding of 2-AAF to liver DNA and hemoglobin in mice and rats was determined in order to test some of the predictions of the pharmacokinetics model. The binding of carcinogens and mutagens to hemoglobin has been proposed as a systemic dose monitor and as a predictor of DNA binding (Ehrenberg and Osterman-Golkar, 1980). The binding of 2-AAF to DNA and hemoglobin was determined to be dose related in a linear manner. This would indicate that over the dose range studied, the formation in the liver of the reactive metabolite (RM) of 2-AAF was also related to dose in a linear manner. Thus, for orally administered 2-AAF, the rate of absorption, distribution, and metabolism would appear to follow first order kinetics. The binding of chemical carcinogens to hemoglobin would appear to predict the binding to DNA in the liver and the systemic dose.

The metabolism of 2-AAF to reactive metabolites and the binding of the metabolites to DNA are some of the parameters that affect interspecies variation in carcinogenesis. The carcinogenic potency (K) can be calculated by the procedure described by Meselson and Russell (1977) as follows:

$$K = [-\ln (1 - I)]/(Dt^{n+1}) \qquad (4)$$

where I is the cumulative single-risk incidence of tumor, D is the dose rate, t is duration of treatment expressed in units of 2 years, and n is the constant between 2 and 4. As recommended by Meselson and Russell (1977), we employed $n = 3$. Using the data for 2-AAF carcinogenicity in rat liver from Albert *et al.* (1972) and in mouse liver from Littlefield *et al.* (1979), we have calculated the carcinogenic potency in rats to be 14.3 and mice to be 5.8×10^{-2} (kg body wt) (day)/μmol dose (Table 2). The greater carcinogenicity of rats compared to mice was qualitatively demonstrated in 2-AAF binding to liver DNA and hemoglobin. However, the DNA binding in rats was only sevenfold greater than that found in mice and the hemoglobin binding in rats was only twofold greater than mice. The 246-fold greater carcinogenicity in rat liver was therefore not reflected in 2-AAF binding to liver DNA and hemoglobin. The discrepancy between the macromolecular binding of 2-AAF and carcinogenic potency could have resulted because DNA binding correlates better with initiation than carcinogenicity. Therefore, while the demonstration that a chemical binds to DNA would indicate carcinogenic activity, the extent of DNA binding does not predict carcinogenic potency, probably because of the numerous intervening steps in the neoplastic progression.

Sex differences in 2-AAF-induced hepatocarcinogenesis has been demonstrated; female mice and male rats have a greater susceptibility to 2-AAF induced hepatocarcinogenesis than male mice and female rats (Miller *et al.*, 1949; Sidransky *et al.*, 1961; Weisburger *et al.*, 1973). No sex difference was observed in the binding of 2-AAF to liver DNA and hemoglobin except for the apparent higher binding to DNA in female mice liver compared to males. While the higher incidence of liver cancer in female mice might correlate to greater DNA binding, the greater cancer incidence in male rats did not correlate to greater DNA binding. The higher incidence of cancer in male rats

has been associated with testosterone (Toh, 1973). The sex difference of 2-AAF-induced hepatocarcinogenesis in rats would not appear to have resulted from sex-related differences in 2-AAF binding to macromolecules, but rather from a subsequent step in the neoplastic progression such as intrinsic promotion by sex steroids.

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